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(54) Title: REGULATION OF HUMAN NEDD1-RELATED PROTEIN

(57) Abstract: Human NEDD1-related protein can be regulated to treat or prevent diseases involving over-proliferation of cells of the central and peripheral nervous system, including, but not limited to, neuronal and glial tumors.

REGULATION OF HUMAN NEDD1-RELATED PROTEIN

TECHNICAL FIELD OF THE INVENTION

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The invention relates to the area of human NEDD1-related proteins and their regulation.

BACKGROUND OF THE INVENTION

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NEDD1 is a 71 kD protein which shares structural similarity in its amino terminal half with the beta subunit of heterotrimeric GTP-binding proteins. NEDD1 is expressed in the early embryonic brain, although low levels of NEDD1 can be detected in various adult tissues, as well as cell lines (Kumar *et al., J. Biol. Chem.*

15   *269*, 11318-26, 1994). NEDD1 is over expressed in an embryonic carcinoma cell line upon differentiation induced by retinoic acid. Expression of recombinant NEDD1 in a variety of cell lines, including a neuroblastoma cell line, suppresses cell growth. Thus, NEDD1 may be involved in the cessation of growth of neuronal cells during differentiation.

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Because of its involvement in neuronal differentiation and its potential for suppressing neoplastic cell growth, there is a need in the art to identify homologs of NEDD1 which can be regulated to provide therapeutic effects.

SUMMARY OF THE INVENTION

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It is an object of the invention to provide reagents and methods of regulating a human NEDD1-related protein. This and other objects of the invention are provided by one or more of the embodiments described below.

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One embodiment of the invention is a NEDD1-related polypeptide comprising an amino acid sequence selected from the group consisting of:

5   amino acid sequences which are at least about 50% identical to the amino acid sequence shown in SEQ ID NO. 2; and

the amino acid sequence shown in SEQ ID NO. 2.

Yet another embodiment of the invention is a method of screening for agents which
10   decrease the activity of NEDD1-related protein. A test compound is contacted with a NEDD1-related polypeptide comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 50% identical to the amino acid
15   sequence shown in SEQ ID NO. 2; and

the amino acid sequence shown in SEQ ID NO. 2.

Binding between the test compound and the NEDD1-related polypeptide is detected.
20   A test compound which binds to the NEDD1-related polypeptide is thereby identified as a potential agent for decreasing the activity of NEDD1-related protein.

Another embodiment of the invention is a method of screening for agents which decrease the activity of NEDD1-related protein. A test compound is contacted with a
25   polynucleotide encoding a NEDD1-related polypeptide, wherein the polynucleotide comprises a nucleotide sequence selected from the group consisting of:

nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO. 1; and

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the nucleotide sequence shown in SEQ ID NO. 1.

Binding of the test compound to the polynucleotide is detected. A test compound which binds to the polynucleotide is identified as a potential agent for decreasing the activity of NEDD1-related protein. The agent can work by decreasing the amount of the NEDD1-related through interacting with the NEDD1-related mRNA.

Another embodiment of the invention is a method of screening for agents which regulate the activity of NEDD1-related protein. A test compound is contacted with a NEDD1-related polypeptide comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 50% identical to the amino acid sequence shown in SEQ ID NO. 2; and

the amino acid sequence shown in SEQ ID NO. 2.

A NEDD1-related activity of the polypeptide is detected. A test compound which increases NEDD1-related activity of the polypeptide relative to NEDD1-related activity in the absence of the test compound is thereby identified as a potential agent for increasing the activity of NEDD1-related protein. A test compound which decreases NEDD1-related activity of the polypeptide relative to NEDD1-related activity in the absence of the test compound is thereby identified as a potential agent for decreasing the activity of NEDD1-related protein.

Even another embodiment of the invention is a method of screening for agents which decrease the activity of NEDD1-related protein. A test compound is contacted with a NEDD1-related product of a polynucleotide which comprises a nucleotide sequence selected from the group consisting of:

nucleotide sequences which are at least about 50% identical to the nucleotide sequence shown in SEQ ID NO. 1; and

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the nucleotide sequence shown in SEQ ID NO. 1.

Binding of the test compound to the NEDD1-related product is detected. A test
compound which binds to the NEDD1-related product is thereby identified as a
potential agent for decreasing the activity of NEDD1-related protein.

Still another embodiment of the invention is a method of reducing the activity of
NEDD1-related protein. A cell is contacted with a reagent which specifically binds
to a polynucleotide encoding a NEDD1-related polypeptide or the product encoded
by the polynucleotide, wherein the polynucleotide comprises a nucleotide sequence
selected from the group consisting of:

nucleotide sequences which are at least about 50% identical to the nucleotide
sequence shown in SEQ ID NO. 1; and

the nucleotide sequence shown in SEQ ID NO. 1.

NEDD1-related activity in the cell is thereby decreased.

The invention thus provides a human NEDD1-related protein which can be regulated
to provide therapeutic effects, as well as used to identify test compounds which may
act as agonists or antagonists of human NEDD1-related gene products.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the DNA-sequence encoding a NEDD1-related polypeptide.

Fig. 2 shows the amino acid sequence deduced from the DNA-sequence of Fig.1.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to an isolated polynucleotide encoding a NEDD1-related polypeptide and being selected from the group consisting of:

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a)   a polynucleotide encoding a NEDD1-related polypeptide comprising an amino acid sequence selected from the group consisting of:

amino acid sequences which are at least about 50% identical to

10   the amino acid sequence shown in SEQ ID NO. 2; and
the amino acid sequence shown in SEQ ID NO. 2.

b)   a polynucleotide comprising the sequence of SEQ ID NO. 1;

15   c)   a polynucleotide which hybridizes under stringent conditions to a poly-
nucleotide specified in (a) and (b);

d)   a polynucleotide the sequence of which deviates from the polynucleotide sequences specified in (a) to (c) due to the degeneration of the genetic code;

20   and

e)   a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d).

25   Furthermore, it has been discovered by the present applicant that NEDD1-related protein, particularly a human NEDD1-related protein, can be regulated to provide therapeutic effects in diseases or disorders involving over-proliferation of cells of the central and peripheral nervous systems, including neuronal and glial tumors. Human NEDD1-related genes also can be used to screen for NEDD1-related agonists and

30   antagonists.

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NEDD1-related Polypeptides

NEDD1-related polypeptides according to the invention comprise the amino acid sequence shown in SEQ ID NO. 2, a portion of that sequence, or a biologically active
5   variant thereof, as defined below. A NEDD1-related polypeptide therefore can be a portion of a NEDD1-related protein, a full-length NEDD1-related protein, or a fusion protein comprising all or a portion of a NEDD1-related protein.

Biologically Active Variants

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NEDD1-related polypeptide variants preferably are biologically active, *i.e.*, can suppress growth when expressed in various cell lines. *See* Kumar *et al.*, 1994. Preferably, naturally or non-naturally occurring NEDD1-related polypeptide variants have amino acid sequences which are at least about 50, preferably about 75, 90, 96,
15   or 98% identical to an amino acid sequence shown in SEQ ID NO. 2 or a fragment thereof. Percent identity between a putative NEDD1-related polypeptide variant and the amino acid sequence of SEQ ID NO. 2 is determined using the Blast2 alignment program.

20   Variations in percent identity can be due, for example, to amino acid substitutions, insertions, or deletions. Amino acid substitutions are defined as one for one amino acid replacements. They are conservative in nature when the substituted amino acid has similar structural and/or chemical properties. Examples of conservative replacements are substitution of a leucine with an isoleucine or valine, an aspartate with a
25   glutamate, or a threonine with a serine.

Amino acid insertions or deletions are changes to or within an amino acid sequence. They typically fall in the range of about 1 to 5 amino acids. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing
30   biological or immunological activity of a NEDD1-related polypeptide can be found using computer programs well known in the art, such as DNASTAR software.

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Whether an amino acid change results in a biologically active NEDD1-related polypeptide can readily be determined by assaying for suppression of cell growth, as described for example, in the specific examples, below.

5 *Fusion Proteins*

Fusion proteins are useful for generating antibodies against NEDD1-related amino acid sequences and for use in various assay systems. For example, fusion proteins can be used to identify proteins which interact with portions of a NEDD1-related
10 polypeptide. Protein affinity chromatography or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage display systems, can be used for this purpose. Such methods are well known in the art and also can be used as drug screens.

15 A NEDD1-related fusion protein comprises two polypeptide segments fused together by means of a peptide bond. The first polypeptide segment comprises at least 5, 6, 8, 10, 25, or 50 or more contiguous amino acids of SEQ ID NO. 2 or from a biologically active variant, such as those described above. The first polypeptide segment also can comprise full-length NEDD1-related protein.
20

The second polypeptide segment can be a full-length protein or a protein fragment. Proteins commonly used in fusion protein construction include β-galactosidase, β-glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horse-
25 radish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Addition-ally, epitope tags are used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding protein (MBP), S-tag, Lex a DNA binding domain (DBD) fusions, GAL4 DNA
30 binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. A fusion protein also can be engineered to contain a cleavage site located between the

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NEDD1-related polypeptide-encoding sequence and the heterologous protein sequence, so that the NEDD1-related polypeptide can be cleaved and purified away from the heterologous moiety.

5　　　A fusion protein can be synthesized chemically, as is known in the art. Preferably, a fusion protein is produced by covalently linking two polypeptide segments or by standard procedures in the art of molecular biology. Recombinant DNA methods can be used to prepare fusion proteins, for example, by making a DNA construct which comprises a coding sequence selected from SEQ ID NO. 1 in proper reading frame

10　　with nucleotides encoding the second polypeptide segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International Corporation (MIC; Watertown,

15　　MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

Identification of Species Homologs

Species homologs of human NEDD1-related polypeptide can be obtained using

20　　NEDD1-related polypeptide polynucleotides (described below) to make suitable probes or primers for screening cDNA expression libraries from other species, such as mice, monkeys, or yeast, identifying cDNAs which encode homologs of NEDD1-related polypeptide, and expressing the cDNAs as is known in the art.

25　　_NEDD1-related Polynucleotides_

A NEDD1-related polynucleotide can be single- or double-stranded and comprises a coding sequence or the complement of a coding sequence for a NEDD1-related polypeptide. A coding sequence for a human NEDD1-related polypeptide is shown

30　　in SEQ ID NO. 1.

Degenerate nucleotide sequences encoding human NEDD1-related polypeptides, as well as homologous nucleotide sequences which are at least about 50, preferably about 75, 90, 96, or 98% identical to the nucleotide sequence shown in SEQ ID NO. 1 also are NEDD1-related polynucleotides. Percent sequence identity between the sequences of two polynucleotides is determined using computer programs such as ALIGN which employ the FASTA algorithm, using an affine gap search with a gap open penalty of -12 and a gap extension penalty of -2. Complementary DNA (cDNA) molecules, species homologs, and variants of NEDD1-related poly-nucleotides which encode biologically active NEDD1-related polypeptides also are NEDD1-related polynucleotides.

Identification of Variants and Homologs of NEDD1-Related Polynucleotides

Variants and homologs of the NEDD1-related polynucleotides described above also are NEDD1-related polynucleotides. Typically, homologous NEDD1-related poly-nucleotide sequences can be identified by hybridization of candidate polynucleotides to known NEDD1-related polynucleotides under stringent conditions, as is known in the art. For example, using the following wash conditions--2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SSC, 0.1% SDS, 50 °C once, 30 minutes; then 2X SSC, room temperature twice, 10 minutes each--homologous sequences can be identified which contain at most about 25-30% basepair mismatches. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Species homologs of the NEDD1-related polynucleotides disclosed herein also can be identified by making suitable probes or primers and screening cDNA expression libraries from other species, such as mice, monkeys, or yeast. Human variants of NEDD1-related polynucleotides can be identified, for example, by screening human cDNA expression libraries. It is well known that the T_m of a double-stranded DNA decreases by 1-1.5 °C with every 1% decrease in homology (Bonner *et al.*, *J. Mol.*

Biol. 81, 123 (1973). Variants of human NEDD1-related polynucleotides or NEDD1-related polynucleotides of other species can therefore be identified by hybridizing a putative homologous NEDD1-related polynucleotide with a polynucleotide having a nucleotide sequence of SEQ ID NO. 1 or the complement thereof to form a test hybrid. The melting temperature of the test hybrid is compared with the melting temperature of a hybrid comprising polynucleotides having perfectly complementary nucleotide sequences, and the number or percent of basepair mismatches within the test hybrid is calculated.

Nucleotide sequences which hybridize to NEDD1-related polynucleotides or their complements following stringent hybridization and/or wash conditions also are NEDD1- related polynucleotides. Stringent wash conditions are well known and understood in the art and are disclosed, for example, in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20°C below the calculated T_m of the hybrid under study. The T_m of a hybrid between a NEDD1-related polynucleotide having a nucleotide sequence shown in SEQ ID NO. 1 or the complement thereof and a polynucleotide sequence which is at least about 50, preferably about 75, 90, 96, or 98% identical to one of those nucleotide sequences can be calculated, for example, using the equation of Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A. 48*, 1390 (1962):

$T_m = 81.5 \,°C - 16.6(\log_{10}[Na^+]) + 0.41(\%G + C) - 0.63(\%formamide) - 600/l)$,
    where l = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65°C, or 50% formamide, 4X SSC at 42°C, or 0.5X SSC, 0.1% SDS at 65°C. Highly stringent wash conditions include, for example, 0.2X SSC at 65°C.

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Preparation of NEDD1-related polynucleotides

A naturally occurring NEDD1-related polynucleotide can be isolated free of other
cellular components such as membrane components, proteins, and lipids.
5   Polynucleotides can be made by a cell and isolated using standard nucleic acid
purification techniques, or synthesized using an amplification technique, such as the
polymerase chain reaction (PCR), or by using an automatic synthesizer. Methods for
isolating polynucleotides are routine and are known in the art. Any such technique
for obtaining a polynucleotide can be used to obtain isolated NEDD1-related
10   polynucleotides. For example, restriction enzymes and probes can be used to isolate
polynucleotide fragments which comprises NEDD1-related   nucleotide sequences.
Isolated polynucleotides are in preparations which are free or at least 70, 80, or 90%
free of other molecules.

15   NEDD1-related cDNA molecules can be made with standard molecular biology
techniques, using NEDD1-related mRNA as a template. NEDD1-related cDNA
molecules can thereafter be replicated using molecular biology techniques known in
the art and disclosed in manuals such as Sambrook _et al._ (1989). An amplification
technique, such as PCR, can be used to obtain additional copies of polynucleotides of
20   the invention, using either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesizes NEDD1-
related polynucleotides. The degeneracy of the genetic code allows alternate nucleo-
tide sequences to be synthesized which will encode a NEDD1-related polypeptide
25   having, for example, an amino acid sequence shown in SEQ ID NO. 2 or a
biologically active variant thereof.

Extending NEDD1-related polynucleotides

30   Various PCR-based methods can be used to extend the nucleic acid sequences
disclosed herein to detect upstream sequences such as promoters and regulatory

elements. For example, restriction-site PCR uses universal primers to retrieve unknown sequence adjacent to a known locus (Sarkar, *PCR Methods Applic. 2*, 318-322, 1993). Genomic DNA is first amplified in the presence of a primer to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR also can be used to amplify or extend sequences using divergent primers based on a known region (Triglia *et al.*, *Nucleic Acids Res. 16*, 8186, 1988). Primers can be designed using commercially available software, such as OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, Minn.), to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68-72 °C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which can be used is capture PCR, which involves PCR amplification of DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA (Lagerstrom *et al.*, *PCR Methods Applic. 1*, 111-119, 1991). In this method, multiple restriction enzyme digestions and ligations also can be used to place an engineered double-stranded sequence into an unknown fragment of the DNA molecule before performing PCR.

Another method which can be used to retrieve unknown sequences is that of Parker *et al.*, *Nucleic Acids Res. 19*, 3055-3060, 1991). Additionally, PCR, nested primers, and PROMOTERFINDER libraries (CLONTECH, Palo Alto, Calif.) can be used to walk genomic DNA (CLONTECH, Palo Alto, Calif.). This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Randomly-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries can be useful for extension of sequence into 5' non-transcribed regulatory regions.

Commercially available capillary electrophoresis systems can be used to analyze the size or confirm the nucleotide sequence of PCR or sequencing products. For example, capillary sequencing can employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity can be converted to electrical signal using appropriate software (e.g. GENOTYPER and Sequence NAVIGATOR, Perkin Elmer), and the entire process from loading of samples to computer analysis and electronic data display can be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

Obtaining NEDD1-related polypeptides

NEDD1-related polypeptides can be obtained, for example, by purification from human cells, by expression of NEDD1-related polynucleotides, or by direct chemical synthesis.

Protein Purification

NEDD1-related polypeptides can be purified from any human cell which expresses the receptor, including host cells which have been transfected with NEDD1-related polynucleotides. A purified NEDD1-related polypeptide is separated from other compounds which normally associate with the NEDD1-related polypeptide in the

cell, such as certain proteins, carbohydrates, or lipids, using methods well-known in the art. Such methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, and preparative gel electrophoresis. A preparation of purified NEDD1-related polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

Expression of NEDD1-related polynucleotides

To express a NEDD1-related polypeptide, a NEDD1-related polynucleotide can be inserted into an expression vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding NEDD1-related polypeptides and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

A variety of expression vector/host systems can be utilized to contain and express sequences encoding a NEDD1-related polypeptide. These include, but are not limited to, microorganisms, such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems.

The control elements or regulatory sequences are those non-translated regions of the vector -- enhancers, promoters, 5' and 3' untranslated regions -- which interact with host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene, LaJolla, Calif.) or pSPORT1 plasmid (Life Technologies) and the like can be used. The baculovirus polyhedrin promoter can be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (*e.g.*, heat shock, RUBISCO, and storage protein genes) or from plant viruses (*e.g.*, viral promoters or leader sequences) can be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of a nucleotide sequence encoding a NEDD1-related polypeptide, vectors based on SV40 or EBV can be used with an appropriate selectable marker.

Bacterial and Yeast Expression Systems

In bacterial systems, a number of expression vectors can be selected depending upon the use intended for the NEDD1-related polypeptide. For example, when a large quantity of a NEDD1-related polypeptide is needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified can be used. Such vectors include, but are not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene). In a BLUESCRIPT vector, a sequence encoding the NEDD1-related polypeptide can be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of β-galactosidase so that a hybrid protein is produced. pIN vectors (Van Heeke & Schuster, *J. Biol. Chem. 264*, 5503-5509, 1989) or pGEX vectors (Promega, Madison, Wis.) also can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion

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proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems can be designed to include heparin, thrombin, or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be

5   released from the GST moiety at will.

In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH can be used. For reviews, see Ausubel *et al.* (1989) and Grant *et al.*, *Methods Enzymol. 153*, 516-

10   544, 1987.

Plant and Insect Expression Systems

If plant expression vectors are used, the expression of sequences encoding NEDD1-

15   related polypeptides can be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV can be used alone or in combination with the omega leader sequence from TMV (Takamatsu, *EMBO J. 6*, 307-311, 1987). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters can be used (Coruzzi *et al.*, *EMBO J. 3*, 1671-

20   1680, 1984; Broglie *et al.*, *Science 224*, 838-843, 1984; Winter *et al.*, *Results Probl. Cell Differ. 17*, 85-105, 1991). These constructs can be introduced into plant cells by direct DNA transformation or by pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (*e.g.*, Hobbs or Murray, in McGraw Hill Yearbook of Science and Technology, McGraw Hill, New York,

25   N.Y., pp. 191-196, 1992).

An insect system also can be used to express a NEDD1-related polypeptide. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells

30   or in *Trichoplusia* larvae. Sequences encoding NEDD1-related polypeptides can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and

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placed under control of the polyhedrin promoter. Successful insertion of NEDD1-related polypeptides will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses can then be used to infect *S. frugiperda cells* or *Trichoplusia* larvae in which NEDD1-related polypeptides can be expressed (Engelhard *et al.*, *Proc. Nat. Acad. Sci. 91*, 3224-3227, 1994).

Mammalian Expression Systems

A number of viral-based expression systems can be used to express NEDD1-related polypeptides in mammalian host cells. For example, if an adenovirus is used as an expression vector, sequences encoding NEDD1-related polypeptides can be ligated into an adenovirus transcription/translation complex comprising the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome can be used to obtain a viable virus which is capable of expressing a NEDD1-related polypeptide in infected host cells (Logan & Shenk, *Proc. Natl. Acad. Sci. 81*, 3655-3659, 1984). If desired, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, can be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) also can be used to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6M to 10M are constructed and delivered to cells via conventional delivery methods (*e.g.*, liposomes, polycationic amino polymers, or vesicles).

Specific initiation signals also can be used to achieve more efficient translation of sequences encoding NEDD1-related polypeptides. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding a NEDD1-related polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding

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sequence, or a fragment thereof, is inserted, exogenous translational control signals (including the ATG initiation codon) should be provided. The initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression can be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used (see Scharf *et al.*, *Results Probl. Cell Differ. 20*, 125-162, 1994).

Host Cells

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed NEDD1-related polypeptide in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the polypeptide also can be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (*e.g.*, CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; 10801 University Boulevard, Manassas, VA 20110-2209) and can be chosen to ensure the correct modification and processing of the foreign protein.

Stable expression is preferred for long-term, high-yield production of recombinant proteins. For example, cell lines which stably express NEDD1-related polypeptides can be transformed using expression vectors which can contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells can be allowed to grow for 1-2 days in an enriched medium before they are switched to a selective medium. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced NEDD1-related sequences. Resistant clones of stably

transformed cells can be proliferated using tissue culture techniques appropriate to the cell type. See, for example, ANIMAL CELL CULTURE, R.I. Freshney, ed., 1986.

Any number of selection systems can be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, *Cell 11*, 223-32, 1977) and adenine phosphoribosyltransferase (Lowy *et al.*, *Cell 22*, 817-23, 1980) genes which can be employed in *tk*⁻ or *aprt*⁻ cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, *dhfr* confers resistance to methotrexate (Wigler *et al.*, *Proc. Natl. Acad. Sci. 77*, 3567-70, 1980), *npt* confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin *et al.*, *J. Mol. Biol. 150*, 1-14, 1981), and *als* and *pat* confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murray, 1992, *supra*). Additional selectable genes have been described. For example, *trpB* allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, *Proc. Natl. Acad. Sci. 85*, 8047-51, 1988). Visible markers such as anthocyanins, β-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, can be used to identify transformants and to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes *et al.*, *Methods Mol. Biol. 55*, 121-131, 1995).

Detecting Expression of NEDD1-related Polypeptides

Although the presence of marker gene expression suggests that the NEDD1-related polynucleotide is also present, its presence and expression may need to be confirmed. For example, if a sequence encoding a NEDD1-related polypeptide is inserted within a marker gene sequence, transformed cells containing sequences which encode a NEDD1-related polypeptide can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a sequence encoding a NEDD1-related polypeptide under the control of a single promoter.

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Expression of the marker gene in response to induction or selection usually indicates expression of the NEDD1-related polynucleotide.

5   Alternatively, host cells which contain a NEDD1-related polynucleotide and which express a NEDD1-related polypeptide can be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip-based technologies for the detection and/or quantification of nucleic acid or protein. For example, the presence
10   of a polynucleotide sequence encoding a NEDD1-related polypeptide can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments or fragments of polynucleotides encoding a NEDD1-related polypeptide. Nucleic acid amplification-based assays involve the use of oligonucleotides selected from sequences encoding a NEDD1-related polypeptide to detect transformants
15   which contain a NEDD1-related polynucleotide.

A variety of protocols for detecting and measuring the expression of a NEDD1-related polypeptide, using either polyclonal or monoclonal antibodies specific for the polypeptide, are known in the art. Examples include enzyme-linked immunosorbent
20   assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay using monoclonal antibodies reactive to two non-interfering epitopes on a NEDD1-related polypeptide can be used, or a competitive binding assay can be employed. These and other assays are described in Hampton et al., SEROLOGICAL METHODS: A LABORATORY MANUAL,
25   APS Press, St. Paul, Minn., 1990) and Maddox et al., J. Exp. Med. 158, 1211-1216, 1983).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for
30   producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding NEDD1-related polypeptides include oligolabeling, nick

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translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, sequences encoding a NEDD1-related polypeptide can be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and can be used to synthesize RNA probes *in vitro* by addition of labeled nucleotides and an appropriate RNA polymerase such as T7, T3, or SP6. These procedures can be conducted using a variety of commercially available kits (Amersham Pharmacia Biotech, Promega, and US Biochemical). Suitable reporter molecules or labels which can be used for ease of detection include radionuclides, enzymes, and fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Expression and Purification of NEDD1-related polypeptides

Host cells transformed with nucleotide sequences encoding a NEDD1-related polypeptide can be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The polypeptide produced by a transformed cell can be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode NEDD1-related polypeptides can be designed to contain signal sequences which direct secretion of soluble NEDD1-related poly-peptides through a prokaryotic or eukaryotic cell membrane or which direct the membrane insertion of membrane-bound NEDD1-related polypeptide.

As discussed above, other constructions can be used to join a sequence encoding a NEDD1-related polypeptide to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). Inclusion of cleavable linker sequences such as those specific for

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Factor Xa or enterokinase (Invitrogen, San Diego, CA) between the purification domain and the NEDD1-related polypeptide also can be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing a NEDD1-related polypeptide and 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification by IMAC (immobilized metal ion affinity chromatography, as described in Porath *et al.*, *Prot. Exp. Purif.* 3, 263-281, 1992), while the enterokinase cleavage site provides a means for purifying the NEDD1-related polypeptide from the fusion protein. Vectors which contain fusion proteins are disclosed in Kroll *et al.*, *DNA Cell Biol.* 12, 441-453, 1993.

Chemical Synthesis

Sequences encoding a NEDD1-related polypeptide can be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers *et al.*, *Nucl. Acids Res. Symp. Ser.* 215-223, 1980; Horn *et al. Nucl. Acids Res. Symp. Ser.* 225-232, 1980). Alternatively, a NEDD1-related polypeptide itself can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc.* 85, 2149-2154, 1963; Roberge *et al.*, *Science* 269, 202-204, 1995). Protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of NEDD1-related polypeptides can be separately synthesized and combined using chemical methods to produce a full-length molecule.

The newly synthesized peptide can be substantially purified by preparative high performance liquid chromatography (*e.g.*, Creighton, PROTEINS: STRUCTURES AND MOLECULAR PRINCIPLES, WH Freeman and Co., New York, N.Y., 1983). The composition of a synthetic NEDD1-related polypeptide can be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; *see* Creighton,

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supra). Additionally, any portion of the amino acid sequence of the NEDD1-related polypeptide can be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion protein.

5

Production of Altered NEDD1-related polypeptides

As will be understood by those of skill in the art, it may be advantageous to produce NEDD1-related polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter NEDD1-related polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

Antibodies

Any type of antibody known in the art can be generated to bind specifically to an epitope of a NEDD1-related polypeptide. "Antibody" as used herein includes intact immunoglobulin molecules, as well as fragments thereof, such as Fab, F(ab')$_2$, and Fv, which are capable of binding an epitope of a NEDD1-related polypeptide. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an

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epitope. However, epitopes which involve non-contiguous amino acids may require more, *e.g.*, at least 15, 25, or 50 amino acids.

An antibody which specifically binds to an epitope of a NEDD1-related polypeptide can be used therapeutically, as well as in immunochemical assays, such as Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immuno-precipitations, or other immunochemical assays known in the art. Various immuno-assays can be used to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays are well known in the art. Such immunoassays typically involve the measurement of complex formation between an immunogen and an antibody which specifically binds to the immunogen.

Typically, an antibody which specifically binds to a NEDD1-related polypeptide provides a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in an immunochemical assay. Preferably, antibodies which specifically bind to NEDD1-related polypeptides do not detect other proteins in immunochemical assays and can immunoprecipitate a NEDD1-related polypeptide from solution.

NEDD1-related polypeptides can be used to immunize a mammal, such as a mouse, rat, rabbit, guinea pig, monkey, or human, to produce polyclonal antibodies. If desired, a NEDD1-related polypeptide can be conjugated to a carrier protein, such as bovine serum albumin, thyroglobulin, and keyhole limpet hemocyanin. Depending on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's adjuvant, mineral gels (*e.g.*, aluminum hydroxide), and surface active substances (*e.g.* lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol). Among adjuvants used in humans, BCG (*bacilli Calmette-Guerin*) and *Corynebacterium parvum* are especially useful.

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Monoclonal antibodies which specifically bind to a NEDD1-related polypeptide can be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These techniques include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the
5   EBV-hybridoma technique (Kohler *et al.*, *Nature 256*, 495-497, 1985; Kozbor *et al.*, *J. Immunol. Methods 81*, 31-42, 1985; Cote *et al.*, *Proc. Natl. Acad. Sci. 80*, 2026-2030, 1983; Cole *et al.*, *Mol. Cell Biol. 62*, 109-120, 1984).

In addition, techniques developed for the production of "chimeric antibodies," the
10 splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity, can be used (Morrison *et al.*, *Proc. Natl. Acad. Sci. 81*, 6851-6855, 1984; Neuberger *et al.*, *Nature 312*, 604-608, 1984; Takeda *et al.*, *Nature 314*, 452-454, 1985). Monoclonal and other antibodies also can be "humanized" to prevent a patient from mounting an immune response
15 against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence differences between rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences by site directed mutagenesis of individual
20 residues or by grating of entire complementarity determining regions. Alternatively, humanized antibodies can be produced using recombinant methods, as described in GB2188638B. Antibodies which specifically bind to a NEDD1-related polypeptide can contain antigen binding sites which are either partially or fully humanized, as disclosed in U.S. 5,565,332.
25

Alternatively, techniques described for the production of single chain antibodies can be adapted using methods known in the art to produce single chain antibodies which specifically bind to NEDD1-related polypeptides. Antibodies with related specificity, but of distinct idiotypic composition, can be generated by chain shuffling from
30 random combinatorial immunoglobin libraries (Burton, *Proc. Natl. Acad. Sci. 88*, 11120-23, 1991).

Single-chain antibodies also can be constructed using a DNA amplification method, such as PCR, using hybridoma cDNA as a template (Thirion *et al.*, 1996, *Eur. J. Cancer Prev. 5*, 507-11). Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught, for example, in Coloma & Morrison, 1997, *Nat. Biotechnol. 15*, 159-63. Construction of bivalent, bispecific single-chain antibodies is taught in Mallender & Voss, 1994, *J. Biol. Chem. 269*, 199-206.

A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant DNA methods, and introduced into a cell to express the coding sequence, as described below. Alternatively, single-chain antibodies can be produced directly using, for example, filamentous phage technology (Verhaar *et al.*, 1995, *Int. J. Cancer 61*, 497-501; Nicholls *et al.*, 1993, *J. Immunol. Meth. 165*, 81-91).

Antibodies which specifically bind to NEDD1-related polypeptides also can be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi *et al.*, *Proc. Natl. Acad. Sci. 86*, 3833-3837, 1989; Winter *et al.*, *Nature 349*, 293-299, 1991).

Other types of antibodies can be constructed and used therapeutically in methods of the invention. For example, chimeric antibodies can be constructed as disclosed in WO 93/03151. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in WO 94/13804, also can be prepared.

Antibodies according to the invention can be purified by methods well known in the art. For example, antibodies can be affinity purified by passage over a column to

which a NEDD1-related polypeptide is bound. The bound antibodies can then be eluted from the column using a buffer with a high salt concentration.

Antisense Oligonucleotides

Antisense oligonucleotides are nucleotide sequences which are complementary to a specific DNA or RNA sequence. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form complexes and block either transcription or translation. Preferably, an antisense oligonucleotide is at least 11 nucleotides in length, but can be at least 12, 15, 20, 25, 30, 35, 40, 45, or 50 or more nucleotides long. Longer sequences also can be used. Antisense oligonucleotide molecules can be provided in a DNA construct and introduced into a cell as described above to decrease the level of NEDD1-related gene products in the cell.

Antisense oligonucleotides can be deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. _See_ Brown, _Meth. Mol. Biol. 20_, 1-8, 1994; Sonveaux, _Meth. Mol. Biol. 26_, 1-72, 1994; Uhlmann _et al._, _Chem. Rev. 90_, 543-583, 1990.

Modifications of NEDD1-related gene expression can be obtained by designing antisense oligonucleotides which will form duplexes to the control, 5', or regulatory regions of the NEDD1-related gene. Oligonucleotides derived from the transcription initiation site, _e.g._, between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double

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helix to open sufficiently for the binding of polymerases, transcription factors, or chaperons. Therapeutic advances using triplex DNA have been described in the literature (*e.g.*, Gee *et al.*, in Huber & Carr, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura Publishing Co., Mt. Kisco, N.Y., 1994). An antisense oligonucleotide also can be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Precise complementarity is not required for successful complex formation between an antisense oligonucleotide and the complementary sequence of a NEDD1-related polynucleotide. Antisense oligonucleotides which comprise, for example, 2, 3, 4, or 5 or more stretches of contiguous nucleotides which are precisely complementary to a NEDD1-related polynucleotide, each separated by a stretch of contiguous nucleotides which are not complementary to adjacent NEDD1-related nucleotides, can provide sufficient targeting specificity for NEDD1-related mRNA. Preferably, each stretch of complementary contiguous nucleotides is at least 4, 5, 6, 7, or 8 or more nucleotides in length. Non-complementary intervening sequences are preferably 1, 2, 3, or 4 nucleotides in length. One skilled in the art can easily use the calculated melting point of an antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular NEDD1-related polynucleotide sequence.

Antisense oligonucleotides can be modified without affecting their ability to hybridize to a NEDD1-related polynucleotide. These modifications can be internal or at one or both ends of the antisense molecule. For example, internucleoside phosphate linkages can be modified by adding cholesteryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases and/or sugars, such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide in which the 3' hydroxyl group or the 5' phosphate group are substituted, also can be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art. *See,*

e.g., Agrawal *et al., Trends Biotechnol. 10*, 152-158, 1992; Uhlmann *et al., Chem. Rev. 90*, 543-584, 1990; Uhlmann *et al., Tetrahedron. Lett. 215*, 3539-3542, 1987.

Ribozymes

Ribozymes are RNA molecules with catalytic activity. *See, e.g.*, Cech, *Science 236*, 1532-1539; 1987; Cech, *Ann. Rev. Biochem. 59*, 543-568; 1990, Cech, *Curr. Opin. Struct. Biol. 2*, 605-609; 1992, Couture & Stinchcomb, *Trends Genet. 12*, 510-515, 1996. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (*e.g.*, Haseloff *et al.*, U.S. Patent 5,641,673). The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Examples include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of specific nucleotide sequences.

The coding sequence of a NEDD1-related polynucleotide can be used to generate ribozymes which will specifically bind to mRNA transcribed from the NEDD1-related polynucleotide. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (*see* Haseloff *et al. Nature 334*, 585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.*, EP 321,201).

Specific ribozyme cleavage sites within a NEDD1-related RNA target can be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target RNA containing the cleavage site can be evaluated for secondary structural

features which may render the target inoperable. Suitability of candidate NEDD1-related RNA targets also can be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays. Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related such that upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

Ribozymes can be introduced into cells as part of a DNA construct. Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce a ribozyme-containing DNA construct into cells in which it is desired to decrease NEDD1-related gene expression. Alternatively, if it is desired that the cells stably retain the DNA construct, the construct can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art. A ribozyme-encoding DNA construct can include transcriptional regulatory elements, such as a promoter element, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of ribozymes in the cells.

As taught in Haseloff et al., U.S. Patent 5,641,673, ribozymes can be engineered so that ribozyme expression will occur in response to factors which induce expression of a target gene. Ribozymes also can be engineered to provide an additional level of regulation, so that destruction of mRNA occurs only when both a ribozyme and a target gene are induced in the cells.

Screening Methods

The invention provides assays for screening test compounds which bind to or modulate the activity of a NEDD1-related polypeptide or a NEDD1-related poly-nucleotide. A test compound preferably binds to a NEDD1-related polypeptide or polynucleotide. More preferably, a test compound decreases or increases by about

10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the test compound.

Test Compounds

Test compounds can be pharmacologic agents already known in the art or can be compounds previously unknown to have any pharmacological activity. The compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced re-combinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer, or small molecule libraries of compounds. *See* Lam, *Anticancer Drug Des. 12*, 145, 1997.

Methods for the synthesis of molecular libraries are well known in the art (*see*, for example, DeWitt *et al., Proc. Natl. Acad. Sci. U.S.A. 90*, 6909, 1993; Erb *et al. Proc. Natl. Acad. Sci. U.S.A. 91*, 11422, 1994; Zuckermann *et al., J. Med. Chem. 37*, 2678, 1994; Cho *et al., Science 261*, 1303, 1993; Carell *et al., Angew. Chem. Int. Ed. Engl. 33*, 2059, 1994; Carell *et al., Angew. Chem. Int. Ed. Engl. 33*, 2061; Gallop *et al., J. Med. Chem. 37*, 1233, 1994). Libraries of compounds can be presented in solution (*see, e.g.,* Houghten, *Biotechniques 13*, 412-421, 1992), or on beads (Lam, *Nature 354*, 82-84, 1991), chips (Fodor, *Nature 364*, 555-556, 1993), bacteria or spores (Ladner, U.S. Patent 5,223,409), plasmids (Cull *et al., Proc. Natl. Acad. Sci. U.S.A. 89*, 1865-1869, 1992), or phage (Scott & Smith, *Science 249*, 386-390, 1990; Devlin, *Science 249*, 404-406, 1990); Cwirla *et al., Proc. Natl. Acad. Sci. 97*, 6378-6382, 1990; Felici, *J. Mol. Biol. 222*, 301-310, 1991; and Ladner, U.S. Patent 5,223,409).

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High Throughput Screening

Test compounds can be screened for the ability to bind to NEDD1-related poly-
peptides or polynucleotides or to affect NEDD1-related activity or NEDD1-related
gene expression using high throughput screening. Using high throughput screening,
many discrete compounds can be tested in parallel so that large numbers of test
compounds can be quickly screened. The most widely established techniques utilize
96-well microtiter plates. The wells of the microtiter plates typically require assay
volumes that range from 50 to 500 µl. In addition to the plates, many instruments,
materials, pipettors, robotics, plate washers, and plate readers are commercially
available to fit the 96-well format.

Alternatively, "free format assays," or assays that have no physical barrier between
samples, can be used. For example, an assay using pigment cells (melanocytes) in a
simple homogeneous assay for combinatorial peptide libraries is described by
Jayawickreme *et al., Proc. Natl. Acad. Sci. U.S.A. 19*, 1614-18 (1994). The cells are
placed under agarose in petri dishes, then beads that carry combinatorial compounds
are placed on the surface of the agarose. The combinatorial compounds are partially
released the compounds from the beads. Active compounds can be visualized as
dark pigment areas because, as the compounds diffuse locally into the gel matrix, the
active compounds cause the cells to change colors.

Another example of a free format assay is described by Chelsky, "Strategies for
Screening Combinatorial Libraries: Novel and Traditional Approaches," reported at
the First Annual Conference of The Society for Biomolecular Screening in
Philadelphia, Pa. (Nov. 7-10, 1995). Chelsky placed a simple homogenous enzyme
assay for carbonic anhydrase inside an agarose gel such that the enzyme in the gel
would cause a color change throughout the gel. Thereafter, beads carrying
combinatorial compounds via a photolinker were placed inside the gel and the

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compounds were partially released by UV-light. Compounds that inhibited the enzyme were observed as local zones of inhibition having less color change.

Yet another example is described by Salmon *et al.*, *Molecular Diversity 2*, 57-63
5   (1996). In this example, combinatorial libraries were screened for compounds that had cytotoxic effects on cancer cells growing in agar.

Another high throughput screening method is described in Beutel *et al.*, U.S. Patent 5,976,813. In this method, test samples are placed in a porous matrix. One or more
10   assay components are then placed within, on top of, or at the bottom of a matrix such as a gel, a plastic sheet, a filter, or other form of easily manipulated solid support. When samples are introduced to the porous matrix they diffuse sufficiently slowly, such that the assays can be performed without the test samples running together.

15   *Binding Assays*

For binding assays, the test compound is preferably a small molecule which binds to a NEDD1-related polypeptide, thereby reducing the normal biological activity of the NEDD1-related polypeptide. Examples of such small molecules include, but are not
20   limited to, small peptides or peptide-like molecules. In binding assays, either the test compound or the NEDD1-related polypeptide can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label, such as horse-radish peroxidase, alkaline phosphatase, or luciferase. Detection of a test compound which is bound to the NEDD1-related polypeptide can then be accomplished, for
25   example, by direct counting of radioemmission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

Alternatively, binding of a test compound to a NEDD1-related polypeptide can be determined without labeling either of the interactants. For example, a micro-
30   physiometer can be used to detect binding of a test compound with a NEDD1-related polypeptide. A microphysiometer (*e.g.*, Cytosensor™) is an analytical instrument

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that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a test compound and a NEDD1-related polypeptide (McConnell *et al.*, *Science 257*, 1906-1912, 1992).

5

Determining the ability of a test compound to bind to a NEDD1-related polypeptide also can be accomplished using a technology such as real-time Bimolecular Interaction Analysis (BIA) (Sjolander & Urbaniczky, *Anal. Chem. 63*, 2338-2345, 1991, and Szabo *et al.*, *Curr. Opin. Struct. Biol. 5*, 699-705, 1995). BIA is a

10 technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore™). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

15 In yet another aspect of the invention, a NEDD1-related polypeptide can be used as a "bait protein" in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent 5,283,317; Zervos *et al.*, *Cell 72*, 223-232, 1993; Madura *et al.*, *J. Biol. Chem. 268*, 12046-12054, 1993; Bartel *et al.*, *Biotechniques 14*, 920-924, 1993; Iwabuchi *et al.*, *Oncogene 8*, 1693-1696, 1993; and Brent WO94/10300), to identify other proteins

20 which bind to or interact with the NEDD1-related polypeptide and modulate its activity.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay

25 utilizes two different DNA constructs. For example, in one construct, polynucleotide encoding a NEDD1-related polypeptide can be fused to a polynucleotide encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct a DNA sequence that encodes an unidentified protein ("prey" or "sample") can be fused to a polynucleotide that codes for the activation domain of the known

30 transcription factor. If the "bait" and the "prey" proteins are able to interact *in vivo* to form an protein-dependent complex, the DNA-binding and activation domains of the

transcription factor are brought into close proximity. This proximity allows
transcription of a reporter gene (*e.g.*, LacZ), which is operably linked to a
transcriptional regulatory site responsive to the transcription factor. Expression of the
reporter gene can be detected, and cell colonies containing the functional
5   transcription factor can be isolated and used to obtain the DNA sequence encoding
the protein which interacts with the NEDD1-related polypeptide.

It may be desirable to immobilize either the NEDD1-related polypeptide (or
polynucleotide) or the test compound to facilitate separation of bound from unbound
10   forms of one or both of the interactants, as well as to accommodate automation of the
assay. Thus, either the NEDD1-related polypeptide (or polynucleotide) or the test
compound can be bound to a solid support. Suitable solid supports include, but are
not limited to, glass or plastic slides, tissue culture plates, microtiter wells, tubes,
silicon chips, or particles such as beads (including, but not limited to, latex,
15   polystyrene, or glass beads). Any method known in the art can be used to attach the
NEDD1-related polypeptide (or polynucleotide) or test compound to a solid support,
including use of covalent and non-covalent linkages, passive absorption, or pairs of
binding moieties attached respectively to the polypeptide (or polynucleotide) or test
compound and the solid support. Test compounds are preferably bound to the solid
20   support in an array, so that the location of individual test compounds can be tracked.
Binding of a test compound to a NEDD1-related polypeptide (or polynucleotide) can
be accomplished in any vessel suitable for containing the reactants. Examples of
such vessels include microtiter plates, test tubes, and microcentrifuge tubes.

25   In one embodiment, the NEDD1-related polypeptide is a fusion protein comprising a
domain that allows the NEDD1-related polypeptide to be bound to a solid support.
For example, glutathione-S-transferase fusion proteins can be adsorbed onto
glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivat-
ized microtiter plates, which are then combined with the test compound or the test
30   compound and the non-adsorbed NEDD1-related polypeptide; the mixture is then
incubated under conditions conducive to complex formation (*e.g.*, at physiological

conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components. Binding of the interactants can be determined either directly or indirectly, as described above. Alternatively, the complexes can be dissociated from the solid support before binding is determined.

Other techniques for immobilizing proteins or polynucleotides on a solid support also can be used in the screening assays of the invention. For example, either a NEDD1-related polypeptide (or polynucleotide) or a test compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated NEDD1-related polypeptides (or polynucleotides) or test compounds can be prepared from biotin-NHS(N-hydroxysuccinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, Ill.) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies which specifically bind to a NEDD1-related polypeptide, polynucleotide, or a test compound, but which do not interfere with a desired binding site, such as the active site of the NEDD1-related polypeptide, can be derivatized to the wells of the plate. Unbound target or protein can be trapped in the wells by antibody conjugation.

Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies which specifically bind to the NEDD1-related polypeptide or test compound, enzyme-linked assays which rely on detecting an activity of the NEDD1-related polypeptide, and SDS gel electrophoresis under non-reducing conditions.

Screening for test compounds which bind to a NEDD1-related polypeptide or polynucleotide also can be carried out in an intact cell. Any cell which comprises a NEDD1-related polypeptide or polynucleotide can be used in a cell-based assay system. A NEDD1-related polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Binding of the test compound to a NEDD1-related polypeptide or polynucleotide is determined as described above.

Functional Assays

Functional assays can be carried out as described in the specific examples, after contacting either a purified NEDD1-related polypeptide or an intact cell with a test compound. A test compound which decreases a functional activity of a human NEDD1-related polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential agent for decreasing NEDD1-related protein activity. A test compound which increases a functional activity by at least about 10, preferably about 50, more preferably about 75, 90, or 100% is identified as a potential agent for increasing NEDD1-related protein activity.

NEDD1-Related Gene Expression

In another embodiment, test compounds which increase or decrease NEDD1-related gene expression are identified. A NEDD1-related polynucleotide is contacted with a test compound, and the expression of an RNA or polypeptide product of the NEDD1-related polynucleotide is determined. The level of expression of appropriate mRNA or polypeptide in the presence of the test compound is compared to the level of expression of mRNA or polypeptide in the absence of the test compound. The test compound can then be identified as a modulator of expression based on this comparison. For example, when expression of mRNA or polypeptide is greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator or enhancer of the mRNA or polypeptide expression. Alternatively, when expression of the mRNA or polypeptide is less in the presence of the test compound than in its absence, the test compound is identified as an inhibitor of the mRNA or polypeptide expression.

The level of NEDD1-related mRNA or polypeptide expression in the cells can be determined by methods well known in the art for detecting mRNA or polypeptide. Either qualitative or quantitative methods can be used. The presence of polypeptide

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products of a NEDD1-related polynucleotide can be determined, for example, using a variety of techniques known in the art, including immunochemical methods such as radioimmunoassay, Western blotting, and immunohistochemistry. Alternatively, polypeptide synthesis can be determined *in vivo*, in a cell culture, or in an *in vitro*
5   translation system by detecting incorporation of labeled amino acids into a NEDD1-related polypeptide.

Such screening can be carried out either in a cell-free assay system or in an intact cell. Any cell which expresses a NEDD1-related polynucleotide can be used in a
10   cell-based assay system. The NEDD1-related polynucleotide can be naturally occurring in the cell or can be introduced using techniques such as those described above. Either a primary culture or an established cell line, such as CHO or human embryonic kidney 293 cells, can be used.

15   *Pharmaceutical Compositions*

The invention also provides pharmaceutical compositions which can be administered to a patient to achieve a therapeutic effect. Pharmaceutical compositions of the invention can comprise, for example, a NEDD1-related polypeptide, NEDD1-related
20   polynucleotide, antibodies which specifically bind to a NEDD1-related polypeptide, or mimetics, agonists, antagonists, or inhibitors of a NEDD1-related polypeptide activity. The compositions can be administered alone or in combination with at least one other agent, such as stabilizing compound, which can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline,
25   buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

In addition to the active ingredients, these pharmaceutical compositions can contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries
30   which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Pharmaceutical compositions of the invention can be

administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents can be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores can be used in conjunction with suitable coatings, such as concentrated sugar solutions, which also can contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments can be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, *i.e.*, dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium

stearate, and, optionally, stabilizers. In soft capsules, the active compounds can be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

5   Pharmaceutical formulations suitable for parenteral administration can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally,

10   suspensions of the active compounds can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers also can be used for delivery. Optionally, the suspension also can contain suitable stabilizers or agents which increase the

15   solubility of the compounds to allow for the preparation of highly concentrated solutions. For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

20   The pharmaceutical compositions of the present invention can be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The pharmaceutical composition can be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric,

25   sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation can be a lyophilized powder which can contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

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Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Maack Publishing Co., Easton, Pa.). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

5

Therapeutic Indications and Methods

10   Human NEDD1-related genes may have a role in the suppression of cell growth and/or division in tumors of the nervous system, such as glioblastomas, astrocytomas, oligodendrogliomas, ependymomas, medulloblastomas, meningeal tumors, vascular tumors, pituitary tumors, tumors of the congenital rests, adnexal tumors, and the like. Thus, levels of NEDD1-related polypeptides can be manipulated to provide

15   therapeutic effects in such diseases. For example, NEDD1-related proteins can be supplied to tumor cells to suppress cell growth and/or division. Alternatively, if increased cell growth and/or division is desired, reagents which suppress the function of NEDD1-related protein can be supplied to cells to increase cell growth and/or division. This invention therefore also encompasses the use of novel agents

20   identified by the screening assays described above. Accordingly, it is within the scope of this invention to use a test compound identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a modulating agent, an antisense nucleic acid molecule, a specific antibody, ribozyme, or a NEDD1-related polypeptide binding molecule) can be used in an

25   animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

30

A reagent which affects NEDD1-related activity can be administered to a human cell, either *in vitro* or *in vivo*, to reduce NEDD1-related activity. The reagent preferably binds to an expression product of a human NEDD1-related gene. If the expression product is a protein, the reagent is preferably an antibody. For treatment of human cells *ex vivo*, an antibody can be added to a preparation of stem cells which have been removed from the body. The cells can then be replaced in the same or another human body, with or without clonal propagation, as is known in the art.

In one embodiment, the reagent is delivered using a liposome. Preferably, the liposome is stable in the animal into which it has been administered for at least about 30 minutes, more preferably for at least about 1 hour, and even more preferably for at least about 24 hours. A liposome comprises a lipid composition that is capable of targeting a reagent, particularly a polynucleotide, to a particular site in an animal, such as a human. Preferably, the lipid composition of the liposome is capable of targeting to a specific organ of an animal, such as the lung, liver, spleen, heart brain, lymph nodes, and skin.

A liposome useful in the present invention comprises a lipid composition that is capable of fusing with the plasma membrane of the targeted cell to deliver its contents to the cell. Preferably, the transfection efficiency of a liposome is about 0.5 µg of DNA per 16 nmole of liposome delivered to about 10^6 cells, more preferably about 1.0 µg of DNA per 16 nmole of liposome delivered to about 10^6 cells, and even more preferably about 2.0 µg of DNA per 16 nmol of liposome delivered to about 10^6 cells. Preferably, a liposome is between about 100 and 500 nm, more preferably between about 150 and 450 nm, and even more preferably between about 200 and 400 nm in diameter.

Suitable liposomes for use in the present invention include those liposomes standardly used in, for example, gene delivery methods known to those of skill in the art. More preferred liposomes include liposomes having a polycationic lipid composition and/or liposomes having a cholesterol backbone conjugated to poly-

ethylene glycol. Optionally, a liposome comprises a compound capable of targeting the liposome to a particular cell type, such as a cell-specific ligand exposed on the outer surface of the liposome.

5   Complexing a liposome with a reagent such as an antisense oligonucleotide or ribozyme can be achieved using methods which are standard in the art (see, for example, U.S. Patent 5,705,151). Preferably, from about 0.1 µg to about 10 µg of polynucleotide is combined with about 8 nmol of liposomes, more preferably from about 0.5 µg to about 5 µg of polynucleotides are combined with about 8 nmol
10   liposomes, and even more preferably about 1.0 µg of polynucleotides is combined with about 8 nmol liposomes.

In another embodiment, antibodies can be delivered to specific tissues *in vivo* using receptor-mediated targeted delivery. Receptor-mediated DNA delivery techniques
15   are taught in, for example, Findeis *et al.* *Trends in Biotechnol.* *11*, 202-05 (1993); Chiou *et al.*, GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER (J.A. Wolff, ed.) (1994); Wu & Wu, *J. Biol. Chem.* *263*, 621-24 (1988); Wu *et al.*, *J. Biol. Chem.* *269*, 542-46 (1994); Zenke *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* *87*, 3655-59 (1990); Wu *et al.*, *J. Biol. Chem.* *266*, 338-42 (1991).

20

Determination of a Therapeutically Effective Dose

The determination of a therapeutically effective dose is well within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of
25   active ingredient which increases or decreases NEDD1-related activity relative to the NEDD1-related activity which occurs in the absence of the therapeutically effective dose.

For any compound, the therapeutically effective dose can be estimated initially either
30   in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model also can be used to determine the appropriate concentration range and

route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

Therapeutic efficacy and toxicity, *e.g.*, ED_{50} (the dose therapeutically effective in 50% of the population) and LD_{50} (the dose lethal to 50% of the population), can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD_{50}/ED_{50}.

Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors which can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

Normal dosage amounts can vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of

polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

If the reagent is a single-chain antibody, polynucleotides encoding the antibody can
5   be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium
10   phosphate-mediated transfection.

Effective *in vivo* dosages of an antibody are in the range of about 5 µg to about 50 µg/kg, about 50 µg to about 5 mg/kg, about 100 µg to about 500 µg/kg of patient body weight, and about 200 to about 250 µg/kg of patient body weight. For
15   administration of polynucleotides encoding single-chain antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 200 ng, 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of DNA.

20   If the expression product is mRNA, the reagent is preferably an antisense oligonucleotide or a ribozyme. Polynucleotides which express antisense oligonucleotides or ribozymes can be introduced into cells by a variety of methods, as described above.

25   Preferably, a reagent reduces expression of a NEDD1-related gene or the activity of a NEDD1-related polypeptide by at least about 10, preferably about 50, more preferably about 75, 90, or 100% relative to the absence of the reagent. The effectiveness of the mechanism chosen to decrease the level of expression of a NEDD1-related gene or the activity of a NEDD1-related polypeptide can be assessed
30   using methods well known in the art, such as hybridization of nucleotide probes to

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NEDD1-related mRNA, quantitative RT-PCR, immunologic detection of a NEDD1-related polypeptide, or measurement of NEDD1-related activity.

5   In any of the embodiments described above, any of the pharmaceutical compositions of the invention can be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy can be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents can act synergistically to effect the treatment or prevention of the various disorders described above. Using this
10   approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

Any of the therapeutic methods described above can be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows,
15   horses, rabbits, monkeys, and most preferably, humans.

Diagnostic Methods

NEDD1-related genes also can be used in diagnostic assays for detecting diseases
20   and abnormalities or susceptibility to diseases and abnormalities related to the presence of mutations in the nucleic acid sequences which encode a NEDD1-related polypeptides.

Differences can be determined between the cDNA or genomic sequence encoding a
25   NEDD1-related polypeptide in individuals afflicted with a disease and in normal individuals. If a mutation is observed in some or all of the afflicted individuals but not in normal individuals, then the mutation is likely to be the causative agent of the disease.

30   Sequence differences between a reference gene and a gene having mutations can be revealed by the direct DNA sequencing method. In addition, cloned DNA segments

can be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer can be used with a double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed

5   by conventional procedures using radiolabeled nucleotides or by automatic sequencing procedures using fluorescent tags.

Genetic testing based on DNA sequence differences can be carried out by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without

10   denaturing agents. Small sequence deletions and insertions can be visualized, for example, by high resolution gel electrophoresis. DNA fragments of different sequences can be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (*see, e.g.*, Myers *et*

15   *al., Science 230,* 1242, 1985). Sequence changes at specific locations can also be revealed by nuclease protection assays, such as RNase and S 1 protection or the chemical cleavage method (*e.g.*, Cotton *et al., Proc. Natl. Acad. Sci. USA 85,* 4397-4401, 1985). Thus, the detection of a specific DNA sequence can be performed by methods such as hybridization, RNase protection, chemical cleavage, direct DNA

20   sequencing or the use of restriction enzymes and Southern blotting of genomic DNA. In addition to direct methods such as gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

Altered levels of a NEDD1-related polypeptide also can be detected in various

25   tissues. Assays used to detect levels of the polypeptides in a body sample, such as blood or a tissue biopsy, derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive binding assays, Western blot analysis, and ELISA assays.

30   All patents and patent applications cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention.

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A more complete understanding can be obtained by reference to the following specific examples which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

5   EXAMPLE 1

Detection of NEDD1-related protein activity and treatment of a neuroblastoma with a NEDD1-related gene product

10   The polynucleotide of SEQ ID NO. 1 is inserted into the expression vector pCEV4 and the expression vector pCEV4-NEDD1-related polypeptide obtained is transfected into neuroblastoma cells. The cells are cultured in RPMI-1640 medium with 10-15% fetal calf serum for seven days. After three to seven days, the number of cells is counted using an automatic cell counter. The number of cells in cultures
15   treated with the above expression vector is compared with the number of cells in cultures without this treatment. The number of cells in cultures treated with the above expression vector is not more than 30% of control, indicating that the polypeptide of SEQ ID NO. 2 has a suppressing effect on neoplastic cell growth.

20   Furthermore the above expression vector is administered to a patient with a neuroblastoma. The patient's neuroblastoma is monitored. The size of the neuroblastoma is decreased.

       EXAMPLE 2
25

Identification of test compounds that bind to NEDD1-related polypeptides

       Purified NEDD1-related polypeptides comprising a glutathione-S-transferase protein and absorbed onto glutathione-derivatized wells of 96-well microtiter plates are
30   contacted with test compounds from a small molecule library at pH 7.0 in a physiological buffer solution. NEDD1-related polypeptides comprise an amino acid

sequence shown in SEQ ID NO. 2. The test compounds comprise a fluorescent tag. The samples are incubated for 5 minutes to one hour. Control samples are incubated in the absence of a test compound.

5   The buffer solution containing the test compounds is washed from the wells. Binding of a test compound to a NEDD1-related polypeptide is detected by fluorescence measurements of the contents of the wells. A test compound which increases the fluorescence in a well by at least 15% relative to fluorescence of a well in which a test compound is not incubated is identified as a compound which binds to
10   an ADO-ribosylation factor-related polypeptide.

EXAMPLE 3

Identification of a test compound which decreases NEDD1-related gene expression
15

A test compound is administered to a culture of CHO cells transfected with a NEDD1-related gene expression construct and incubated at 37 °C for 10 to 45 minutes. A culture of the same type of cells incubated for the same time without the test compound provides a negative control.
20

RNA is isolated from the two cultures as described in Chirgwin *et al.*, *Biochem. 18*, 5294-99, 1979). Northern blots are prepared using 20 to 30 μg total RNA and hybridized with a ^{32}P-labeled NEDD1-related gene-specific probe at 65 ° C in Express-hyb (CLONTECH). The probe comprises at least 11 contiguous nucleotides
25   selected from the complement of SEQ ID NO. 1. A test compound which decreases the NEDD1-related gene-specific signal relative to the signal obtained in the absence of the test compound is identified as an inhibitor of NEDD1-related gene expression.

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EXAMPLE 4

Suppression of a test compound on NEDD1-related protein-suppressed cell growth in neuroblastoma cells

5

Neuroblastoma cells are contacted with a test compound and a NEDD1-related protein comprising the amino acid sequence shown in SEQ ID NO. 2. Growth of the neuroblastoma cells is monitored. A test compound which suppresses the ability of NEDD1-related protein to suppress cell growth is identified as a potential reagent for

10 suppressing NEDD1-related protein function. A test compound which increases the ability of NEDD1-related protein to suppress cell growth is identified as a potential reagent for increasing NEDD1-related protein function.

CLAIMS

1.   An isolated polynucleotide encoding a NEDD1-related polypeptide and being selected from the group consisting of:

5

    a)   a polynucleotide encoding a NEDD1-related polypeptide comprising an amino acid sequence selected from the group consisting of:

    amino acid sequences which are at least about 50% identical to

10   the amino acid sequence shown in SEQ ID NO. 2; and
    the amino acid sequence shown in SEQ ID NO. 2.

    b)   a polynucleotide comprising the sequence of SEQ ID NO. 1;

15   c)   a polynucleotide which hybridizes under stringent conditions to a polynucleotide specified in (a) and (b);

    d)   a polynucleotide the sequence of which deviates from the poly-nucleotide sequences specified in (a) to (c) due to the degeneration of

20   the genetic code; and

    e)   a polynucleotide which represents a fragment, derivative or allelic variation of a polynucleotide sequence specified in (a) to (d).

25   2.   An expression vector containing any polynucleotide of claim 1.

3.   A host cell containing the expression vector of claim 2.

4.   A substantially purified NEDD1-related polypeptide encoded by a

30   polynucleotide of claim 1.

5.   A method for producing a NEDD1-related polypeptide, wherein the method comprises the following steps:

　　　　a)   culturing the host cell of claim 3 under conditions suitable for the expression of the NEDD1-related polypeptide; and

　　　　b)   recovering the NEDD1-related polypeptide from the host cell culture.

6.   A method for detection of a polynucleotide encoding a NEDD1-related polypetide in a biological sample comprising the following steps:

　　　　a)   hybridizing any polynucleotide of claim 1 to a nucleic acid material of a biological sample, thereby forming a hybridization complex; and

　　　　b)   detecting said hybridization complex.

7.   The method of claim 6, wherein before hybridization, the nucleic acid material of the biological sample is amplified.

8.   A method for the detection of a polynucleotide of claim 1 or a NEDD1-related polypeptide of claim 5 comprising the steps of contacting a biological sample with a reagent which specifically interacts with the polynucleotide or the NEDD1-related polypeptide.

9.   A diagnostic kit for conducting the method of any one of claims 6 to 8.

10.   A method of screening for agents which decrease the activity of a NEDD1-related protein, comprising the steps of:

　　　　contacting a test compound with any NEDD1-related polypeptide encoded by any polynucleotide of claim 1;

detecting binding of the test compound of the NEDD1-related polypeptide, wherein a test compound which binds to the polypeptide is identified as a potential therapeutic agent for decreasing the activity of a NEDD1-related

5   protein.

11.   A method of screening for agents which regulate the activity of a NEDD1-related protein, comprising the steps of:

10   contacting a test compound with a NEDD1-related polypeptide encoded by any polynucleotide of claim 1; and

         detecting a NEDD1-related activity of the polypeptide, wherein a test compound which increases the NEDD1-related activity is identified as a

15   potential therapeutic agent for increasing the activity of the NEDD1-related protein, and wherein a test compound which decreases the NEDD1-related activity of the polypeptide is identified as a potential therapeutic agent for decreasing the activity of the NEDD1-related protein.

20 12.   A method of screening for agents which decrease the activity of a NEDD1-related protein, comprising the steps of:

         contacting a test compound with any polynucleotide of claim 1 and

25   detecting binding of the test compound to the polynucleotide, wherein a test compound which binds to the polynucleotide is identified as a potential therapeutic agent for decreasing the activity of NEDD1-related protein.

13.   A method of reducing the activity of NEDD1-related protein, comprising the
30   steps of:

contacting a cell with a reagent which specifically binds to any poly-nucleotide of claim 1 or any NEDD1-related polypeptide of claim 4, whereby the activity of NEDD1-related protein is reduced.

5   14.   A reagent that modulates the activity of a NEDD1-related polypeptide or a polynucleotide wherein said reagent is identified by the method of any of the claims 10 to 12.

     15.   A pharmaceutical composition, comprising:
10   the expression vector of claim 2 or the reagent of claim 14 and a pharma-ceutically acceptable carrier.

     16.   Use of the pharmaceutical composition of claim 15 for modulating the activity of a NEDD1-related protein in a disease.

15
     17.   Use of claim 16 wherein the disease includes over-proliferation of cells of the central and peripheral nervous system.

Fig. 1

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ggtacttcag  tattgcattc  tattcctctt  aatgttttta  tgggatctcc
agggaaagag
gaaaatgaaa  accgtgatct  aacagctgag  tctaagaaaa  tatatatggg
aaaacaggaa
tctaaagact  ccttcaaaca  gttagcaaag  ttggtcacat  ctggtgctga
aagtggaaat
ctaaatacct  ctccatcatc  taaccaaaca  agaaattctg  agaaatttga
aaagccagag
aatgaaattg  aagcccagtt  gatatgtgaa  cccccaatca  atggatcctc
aactccaaat
ccaaagatag  catcttctgt  cactgctgga  gttgccagtt  cactctcaga
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Fig. 2

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L T S I Q I R L F K H D Q E T L D D F K K X A X
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SEQUENZPROTOKOLL

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Lys Ile Tyr Met Gly Lys Gln Glu Ser Lys Asp Ser Phe Lys Gln Leu
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Ala Lys Leu Val Thr Ser Gly Ala Glu Ser Gly Asn Leu Asn Thr Ser
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Pro Ser Ser Asn Gln Thr Arg Asn Ser Glu Lys Phe Glu Lys Pro Glu
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Cys Arg Trp Glu Asp Xaa Gln Phe Ile Ser Thr Glu Xaa Asn Ala Ser
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Lys Glu Thr Lys Lys Tyr Arg Pro Leu Xaa Asn Ser Gly Xaa Pro Met
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